

## New Insights into the Anti-inflammatory Mechanisms of Glucocorticoids: An Emerging Role for Glucocorticoid-Receptor-Mediated Transactivation

Sofie Vandevyver, Lien Dejager, Jan Tuckermann, and Claude Libert

Department for Molecular Biomedical Research (S.V., L.D., C.L.), Flanders Institute for Biotechnology, and Department of Biomedical Molecular Biology (S.V., L.D., C.L.), B9052 Ghent, Belgium; and Institute for General Zoology and Endocrinology (J.T.), University of Ulm, D-89081 Ulm, Germany

Glucocorticoids are anti-inflammatory drugs that are widely used for the treatment of numerous (autoimmune) inflammatory diseases. They exert their actions by binding to the glucocorticoid receptor (GR), a member of the nuclear receptor family of transcription factors. Upon ligand binding, the GR translocates to the nucleus, where it acts either as a homodimeric transcription factor that binds glucocorticoid response elements (GREs) in promoter regions of glucocorticoid (GC)-inducible genes, or as a monomeric protein that cooperates with other transcription factors to affect transcription. For decades, it has generally been believed that the undesirable side effects of GC therapy are induced by dimer-mediated transactivation, whereas its beneficial anti-inflammatory effects are mainly due to the monomer-mediated transrepressive actions of GR. Therefore, current research is focused on the development of dissociated compounds that exert only the GR monomer-dependent actions. However, many recent reports undermine this dogma by clearly showing that GR dimer-dependent transactivation is essential in the anti-inflammatory activities of GR. Many of these studies used GR<sup>dim/dim</sup> mutant mice, which show reduced GR dimerization and hence cannot control inflammation in several disease models. Here, we review the importance of GR dimers in the anti-inflammatory actions of GCs/GR, and hence we question the central dogma. We summarize the contribution of various GR dimer-inducible anti-inflammatory genes and question the use of selective GR agonists as therapeutic agents. (*Endocrinology* 154: 993–1007, 2013)

**G**lucocorticoids (GCs) are critical regulators of a wide variety of fundamental processes, including metabolic homeostasis, cell proliferation, inflammatory and immune responses, development, and reproduction (1–3). At pharmacologic concentrations, GCs display potent anti-inflammatory effects. Hence, numerous autoimmune, inflammatory, and allergic disorders, such as asthma, rheumatoid arthritis, ulcerative colitis, and allergic rhinitis (4, 5), are often treated with synthetic GCs, such as dexamethasone and prednisolone. Despite their excellent anti-inflammatory efficacy, the use of GCs as therapeutics is often restrained due to two major drawbacks. First, long-term treatment with GCs is often accompanied by

severe side effects, such as diabetes, osteoporosis, hypertension, and muscle atrophy (6, 7). Second, the occurrence of GC resistance also limits the success of many GC-based therapies.

GCs exert their functions by binding to their intracellular receptor, the glucocorticoid receptor (GR), which is a ligand-inducible transcription factor belonging to the nuclear receptor superfamily (8). The GR is a modular protein composed of three major functional domains: the N-terminal domain, the central DNA-binding domain (DBD), and the C-terminal ligand-binding domain (LBD). The DBD consists of two zinc fingers important for GR dimerization, nuclear translocation, and DNA binding.

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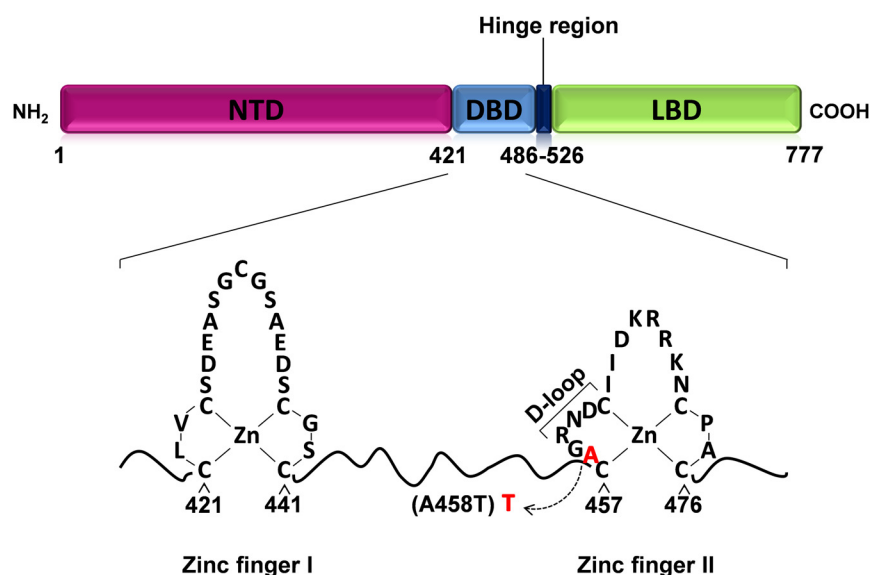
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Abbreviations: AIA, antigen-induced arthritis; AP-1, activator protein 1; CIA, collagen-induced arthritis; COX2, cyclooxygenase 2; DBD, DNA-binding domain; DNBS, dinitrobenzene sulfonic acid; GC, glucocorticoid; GILZ, GC-induced leucine zipper; GR, glucocorticoid receptor; GRE, GR response element; JNK, c-jun N-terminal kinase; LBD, ligand-binding domain; LPS, lipopolysaccharide; MR, mineralocorticoid receptor; NF- $\kappa$ B, nuclear factor- $\kappa$ B; nGRE, negative GRE; SEGRAs, selective GR agonists; TA, transactivation; TR, transrepression.



**Figure 1.** General Structure of the GR (human) is composed of an N-terminal domain (NTD), a DBD, a hinge region, and a C-terminal LBD. The GR DBD contains two zinc fingers in which the zinc molecule is located between four cysteine residues. In GR<sup>dim/dim</sup> mice A458 (red) is mutated to a threonine. The mutant GR cannot form dimers.

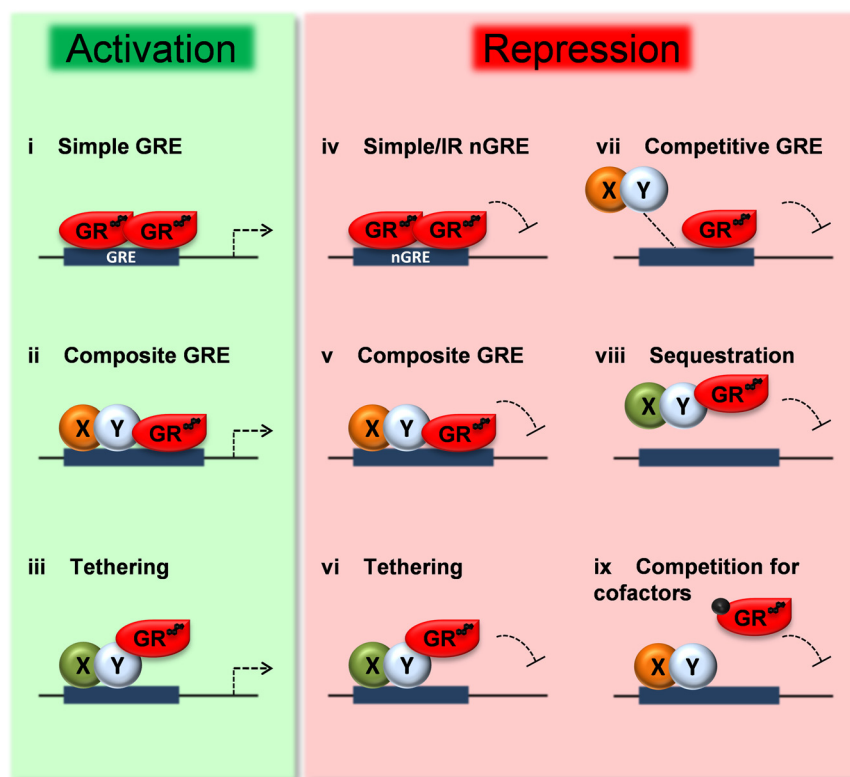
Each zinc finger contains a zinc atom between four cysteine residues. The second zinc finger is more important for GR dimerization. The DBD and LBD are linked by a hinge region, which allows nuclear translocation of GR (9–11) (Figure 1, upper panel). Additionally, GCs can also bind to another nuclear receptor, the mineralocorticoid receptor (MR), with a 10-fold higher affinity than with GR (12), but interference of GCs in MR signaling is limited due to the topical restriction of MR expression. Whereas GR is widely expressed, MR is expressed only in certain types of cells (and regulates salt and water homeostasis). Furthermore, the action of GCs through the MR is limited by the activity of 11 $\beta$ -hydroxysteroid dehydrogenase 2 in cells in which MR is expressed (13, 14).

In its inactive state, GR resides predominantly in the cytoplasm, where it is sequestered in a multimeric chaperone complex consisting of heat shock proteins (such as hsp90, hsp70, hsp90 binding protein p23), immunophilins (eg, FKBP51, FKBP52, Cyp44, and PP5), and other factors to prevent its degradation and to assist in its maturation (15–17). The GR is constitutively expressed in virtually all cell types, but the different tissue-specific patterns lead to tissue-specific outcomes in different diseases (18, 19). Furthermore, GR-mediated effects are readily influenced by epigenetic regulators, context, and other unrecognized determinants (20, 21). In addition, the key variables that determine the GR-mediated outcome include timing and genomic accessibility of GC-responsive genes. The nature and magnitude of a cell's response to GCs also depend on the levels of hormones secreted by the

adrenal gland in a circadian rhythm and undergo pulsatile secretion (22–24).

Although inactive GR is found primarily in the cytoplasm, it is not rigidly compartmentalized. GR shuttles continuously between the nucleus and the cytoplasm through the nuclear pore channel (reviewed in Ref. 15). Nevertheless, upon ligand binding, GR undergoes conformational changes and is mainly found in the nucleus due to its ligand-induced nuclear translocation. In the nucleus, GR mediates the up-regulation of numerous genes and down-regulation of others in a coordinated fashion. Positive regulation is often mediated by the binding of GR to GR-binding sites. The best-described mechanism of transcriptional activation is the direct binding of GR homodimers to

so-called GR response elements (GREs) in the promoter regions of GC-inducible genes (25). In fact, GR homodimers can bind in the major groove of DNA via their DBD-containing two zinc fingers and thus target a GRE (5, 26). The consensus GRE sequence is an inverted imperfect hexameric palindrome separated by a spacer of 3 bp (5'-AGAACA<sub>n</sub>TTGTTCT-3', in which "n" is any nucleotide) (5, 27). The sequence of the GRE varies among different promoters, and therefore the GRE can be considered as a sequence-specific allosteric ligand directing the transcriptional activity of GR (28, 29). However, recent global ChIP-Seq data reveal that GR binds to DNA mostly via the GRE consensus motif (30). Additionally, next to transactivation (TA) of "simple" GRE motifs by GR dimers, GR can cooperate with other transcription factors as a monomeric protein to induce transcription (31, 32) to so-called "composite" elements or by a "tethering" mechanism. Binding of GR to DNA leads to recruitment of distinct cofactors that enable chromatin remodeling, RNA polymerase II binding, and subsequent gene induction. The mechanisms of GR-mediated transcriptional repression or transrepression (TR), on the other hand, are more promiscuous and partly involve DNA binding of homodimeric GR to simple negative GREs (nGREs) or inverted repeats (IR) with less than three spacers to specifically repress gene transcription (33–35). Furthermore, GC-activated monomeric GR can negatively regulate gene transcription, eg, by tethering other transcription factors, such as nuclear factor- $\kappa$ B (NF- $\kappa$ B) and activator protein 1 (AP-1), or through cross-talk with



**Figure 2.** GR Signaling Activated GR can lead to either activation or repression of gene transcription. Left green panel: TA is mediated by (i) binding of GR dimers to GRE, (ii) DNA binding of GR in concert with another transcription factor (TF: XY), or (iii) binding of GR to a TF by a tethering mechanism. Right red panel: TR is mainly achieved by (iv) direct binding of GR dimers to nGRE (simple or IR), (v) DNA-binding cross-talk with another TF, (vi) interference of monomeric GR with the TA activity of TFs by a tethering mechanism, (vii) competition for an overlapping binding site (competitive GRE), (viii) sequestration of a DNA-bound TF, or (ix) competition for binding cofactors with other DNA-bound TFs.

other transcription factors and binding to “composite” elements (36, 37). For an overview of the fundamental aspects of GR transcriptional regulation, see Figure 2.

The anti-inflammatory effects of GR are believed to generally result from tethering protein-protein interactions between GR and other transcription factors, particularly NF- $\kappa$ B and AP-1, which results in TR of a wide variety of proinflammatory genes. On the other hand, the debilitating GC-mediated effects are thought to be caused by TA of simple GRE genes (38, 39). Accordingly, so-called selective GR agonists (SEGRAs) that favor TR were developed as therapeutic agents with reduced side effects. Examples are RU24858, compound A, AL-438, LGD5552, and ZK 216348 (40–45). However, more recent data show that the TA potential of GR is indispensable for its anti-inflammatory properties, at least in certain disease settings. Here, we provide an overview of the anti-inflammatory mechanisms of GR, focusing mainly on the induction of anti-inflammatory genes by GR as a homodimeric transcription factor and with emphasis on *in vivo* studies.

## Current Concept of the Anti-inflammatory Mechanism of GC/GR: Emphasis on TR

Until recently, it was generally believed that TR of transcription factors by monomeric GR is the main determinant of the anti-inflammatory properties of GR, whereas its side effects reside in its TA potential (36, 38, 39, 46). This concept has been reviewed extensively (31, 41, 47). Briefly, it is known that TA, through direct DNA binding, induces the expression of several enzymes (eg, phosphoenol pyruvate carboxykinase, tyrosine aminotransferase, and glucose 6-phosphate) involved in glucose and lipid metabolism. Hence, uncontrolled up-regulation of these genes could account for the diabetogenic effects of GCs, which result in hyperglycemia and decreased carbohydrate tolerance (1, 48). On the other hand, it is believed that the anti-inflammatory actions of GC therapy are predominantly related to the TR effects of GR (11, 49) because some inflammatory processes could still be restricted in a

mouse strain (GR<sup>dim/dim</sup>) in which GR is largely dimerization defective due to replacement of an alanine by a threonine (A458T) (11, 50, 51). This mutation is located in the second zinc finger in the DBD of GR (Figure 1, lower panel) and causes reduced binding to DNA and, more specifically, to the GRE (11, 50, 51).

TR of inflammatory target genes most often involves interference of GR with the activity of DNA-bound proinflammatory transcription factors, such as NF- $\kappa$ B, cAMP response element-binding protein, interferon regulating factor-3, nuclear factor of activated T cells, signal transducers and activators of transcription, Th1-specific T box transcription factor, GATA3, and AP-1 (52–54). Because these transcription factors regulate the expression of inflammatory genes, GR-mediated tethering of these transcription factors eventually leads to repression of a large number of proinflammatory mediators: cytokines (including TNF, granulocyte macrophage colony stimulating factor, IL-1 $\beta$ , IL-2, IL-3, and IL-6), chemokines (eg, eotaxin, macrophage inflammatory protein [MIP], and regulated and normal T cell expressed and secreted [RANTES]), en-

zymes (such as inducible nitric oxide synthase and cyclooxygenase 2 [COX2]), and adhesion molecules (eg, intercellular adhesion molecule 1 and vascular cell adhesion molecule 1). Thus, negative regulation by tethering has become a paradigm for the anti-inflammatory and immune-suppressive actions of GR. The most-studied cross-talk mechanisms are those between GR and NF- $\kappa$ B, GR and AP-1, and GR and interferon regulating factor-3 (41, 42, 45, 55).

### Studies on GR<sup>dim/dim</sup> Mice: The Emerging Role of GR Dimerization in the Anti-inflammatory Function of GR

As mentioned above, most studies claim that interaction of monomeric GR with proinflammatory transcription factors is the basis of its anti-inflammatory activity. However, the contribution of GR dimers to its anti-inflammatory property remains controversial. Mounting evidence indicates that the TA potential of GR dimers is required for execution of the complete anti-inflammatory cascade (49, 56–59). Most studies involved experiments in GR<sup>dim/dim</sup> mice.

Importantly, GRE-dependent gene transcription is diminished in cells originating from GR<sup>dim/dim</sup> mice, as shown by impaired induction of a mouse mammary tumor virus-CAT reporter in GR<sup>dim/dim</sup> mouse embryo fibroblast cells and GR-inducible gluconeogenic enzymes, such as *g6p* and *pck1*, in liver lysates of GR<sup>dim/dim</sup> mice and reduced (but not absent) GC regulation of genes in the liver as revealed by genome-wide expression profiling (10, 11). However, the repressing function (cross-talk with AP-1 and NF- $\kappa$ B) of GR is still intact in GR<sup>dim/dim</sup> mice (11, 49, 51, 60).

Almost all evidence discussed in this review is based on the analysis of the single-point mutant A458T, otherwise known as the GR<sup>dim/dim</sup> mutant. However, one should consider these data with caution, because mounting evidence indicates that additional residues are indispensable for GR dimerization. The idea that GR<sup>dim/dim</sup> mutants cannot form dimers has been challenged by a recent study (61). Human osteosarcoma (U-2 OS) cells expressing the GR<sup>wt/wt</sup> receptor or the hGR<sup>dim/dim</sup> (A458T) or hGR<sup>dim4</sup> (A458T, R460D, D462C, and N454D) mutant were used with GRE-driven reporters in transient reporter gene assays. The results revealed that the hGR<sup>dim4</sup> mutant is even more unresponsive to steroids than the hGR<sup>dim/dim</sup> (A458T) mutant. These findings are in agreement with the resistance of human cell lines carrying these mutations to GC-mediated apoptosis (61). These findings are in line with earlier published data showing that both the GR<sup>dim/dim</sup>

and GR<sup>dim4</sup> mutations in the D-loop strongly inhibited GR dimerization and GR-mediated TA but did not hamper the repression of AP-1 and NF- $\kappa$ B (51). Indeed, Jewell et al. (61) also showed that the TR capacity of hGR<sup>dim/dim</sup> was indeed unaffected. What is particularly interesting is that immunoprecipitation experiments showed that both the human (h)GR<sup>dim/dim</sup> and hGR<sup>dim4</sup> receptors could promptly form dimers. Of course, these striking findings should be experimentally confirmed further. Furthermore, Savory et al. (62) have demonstrated a novel dimer interface in the LBD of GR. Mutating this dimer interface's most important residues (P625 and I628) to alanines resulted in a 10-fold decrease in dimerization affinity relative to wild-type (WT) LBD. Furthermore, by using a mouse mammary tumor virus reporter assay, Bledsoe et al. (63) showed that the residues of this dimer interface are also important for the GR TA function. In addition, more recent gene expression profiling by Frijters (10 unambiguously confirms that the GR<sup>dim/dim</sup> mutant can still transactivate some genes, albeit not as strongly as its wild type counterpart.

Taken together, all the above-mentioned findings indicate that the single-point mutation in the DBD, namely A458T in the GR<sup>dim/dim</sup> mutant, may not be sufficient to completely abolish dimerization and thus GR-mediated TA. Most probably, the GR<sup>dim/dim</sup> mutant can still bind to a set of GR-responsive promoters, although in a cell type- and gene promoter-specific manner, by forming multimers independently of the DBD-dimer interface. Nevertheless, all studies performed with the GR<sup>dim/dim</sup> mutant and subsequent findings do prove that this single-point mutation reduces dimerization, and that the GR-DNA binding potential and TA are critically important in the anti-inflammatory actions of GR.

Mice carrying the GR<sup>dim/dim</sup> mutation (11) are viable, in contrast to the full GR knock-out mouse (64). GR<sup>dim/dim</sup> mice are normal in size and appear normal, but they do show some anomalies, such as increased expression of *Pomc* in the pituitary gland, which demonstrates the loss of negative control of *Pomc* transcription by GR dimers. This results in elevated levels of secreted ACTH and GCs (11, 65). Additionally, studies on GR<sup>dim/dim</sup> mice revealed that GR dimerization is required for GC-mediated thymocyte apoptosis and long-term proliferation of erythroblasts (11). Another cellular process that necessitates action of GR dimers is adipogenesis: this process could be promoted by induction of Krüppel-like factor 15 by a dimerized GR (66). Furthermore, GR dimers are also required for the task-related facilitating effects of GCs on spatial memory (65). In contrast, dimerization of GR is dispensable for epidermal and hair follicle development during embryogenesis (67).



**Table 1.** Identification of the role of GR dimers in different physiological responses to GCs by using GR<sup>dim/dim</sup> mice

Effects	GR Dimerization	Important Cell Types	References
Resolution of inflammation			
Antigen- and G6PI-induced arthritis	Required	IL-17 producing T-cells	(56)
Contact hypersensitivity	Required	Macrophages Neutrophils	(58)
LPS- and CLP-induced septic shock	Required	Macrophages	(57)
PMA-induced irritative skin inflammation	Dispensable	T-lymphocytes Macrophages	(49, 60)
TNF-induced inflammation	Required	Intestinal epithelial cells	(59)
Side effects			
Hyperglycemia	Required	Enterocytes	(124)
Osteoporosis	Dispensable	Osteoblasts Osteoclasts	(85, 125)
Skeletal muscle atrophy	Dispensable	Unknown	(126)
Wound repair	Required	Unknown	(123)
Cellular processes			
Adipogenesis	Required	Fibroblasts	(66)
Apoptosis	Required	Thymocytes	(11, 61)
Epidermal development during embryogenesis	Dispensable	Unknown	(67)
Proliferation	Required	Erythroblasts	(11)
Spatial memory	Required	Unknown	(65)

CLP, cecal ligation and puncture; PMA, phorbol myristate acetate.

The contribution of the DNA-binding function of GR to the anti-inflammatory effects of GR was until recently strongly underestimated. Several studies have exploited the response of GR<sup>dim/dim</sup> mice in several inflammatory disease models (Table 1). It has been shown that DNA-binding GR dimers are not required in GC therapy of irritative skin inflammation induced by phorbol ester (phorbol myristate acetate), and that GR monomers can inhibit inflammation in this model (49, 60). In contrast, GR<sup>dim/dim</sup> mice were refractory to GC treatment in a mouse model of contact hypersensitivity, which mimics allergic contact dermatitis (58). These data indicate that dimerization of GR and subsequent GRE-dependent transcription are indispensable for the restriction of certain allergic inflammatory disorders. Similar to contact hypersensitivity, Baschant et al. (56) showed that GCs require GR dimer activity to restrain inflammation in two murine rheumatoid arthritis models: antigen-induced arthritis (AIA) and glucose-6-phosphate isomerase-induced arthritis. GC treatment did not protect GR<sup>dim/dim</sup> mice, indicating that the DNA-binding capacity of dimeric GR is required for GC-mediated suppression of arthritis inflammation. Moreover, GR<sup>dim/dim</sup> mice are also highly susceptible in several septic shock models, such as sepsis induced by lipopolysaccharide (LPS) or cecal ligation and puncture, and inflammation induced by TNF (57, 59). These studies provide evidence that in septic shock, the therapeutic actions of endogenous and exogenous applied GCs require GR dimerization. Taken together, these data undermine the concept that GR monomers are responsible for most of the anti-inflammatory potential of GCs and clearly show that GR dimer-dependent TA is essential for the anti-inflammatory activities of GR.

## GR Dimer-Dependent Transcriptional Actions

### GR-mediated TA of anti-inflammatory genes

Gene expression profiling of livers of GR<sup>wt/wt</sup> and GR<sup>dim/dim</sup> mice treated with prednisolone revealed that many genes could not be significantly induced in GR<sup>dim/dim</sup> mice, indicating that their induction depends on GR dimers (10). Many of these genes have well-known anti-inflammatory actions and, hence, might contribute to the anti-inflammatory properties of GR. Here, we will focus on a few prominent genes and elaborate on their anti-inflammatory actions. A complete overview of all GC-inducible anti-inflammatory genes identified so far and their effects on the proinflammatory cascade are depicted in Table 2 and Figure 3.

Whereas *Dusp1*, *Tsc22d3*, and *Anxa1* are only just a few GC-inducible genes mediating some of the anti-inflammatory capacities of GR, microarray profiling data indicate that many other genes are positively regulated by GR and play a putative role in the dispute against inflammation (10, 57, 68). However, identification of these genes and their functionality is still in its infancy, which means that the complexity of the anti-inflammatory nature of GR is still far from fully understood.

### MAPK phosphatase 1 or dual specificity phosphatase 1 (MKP-1 or *Dusp1*)

Dual specificity phosphatase 1 (encoded by *Dusp1*) is one of the most potent anti-inflammatory GR-inducible proteins. It is a member of the dual-specificity phosphatases, which include 10 members, and catalyzes the dephosphorylation and subsequent inactivation of both

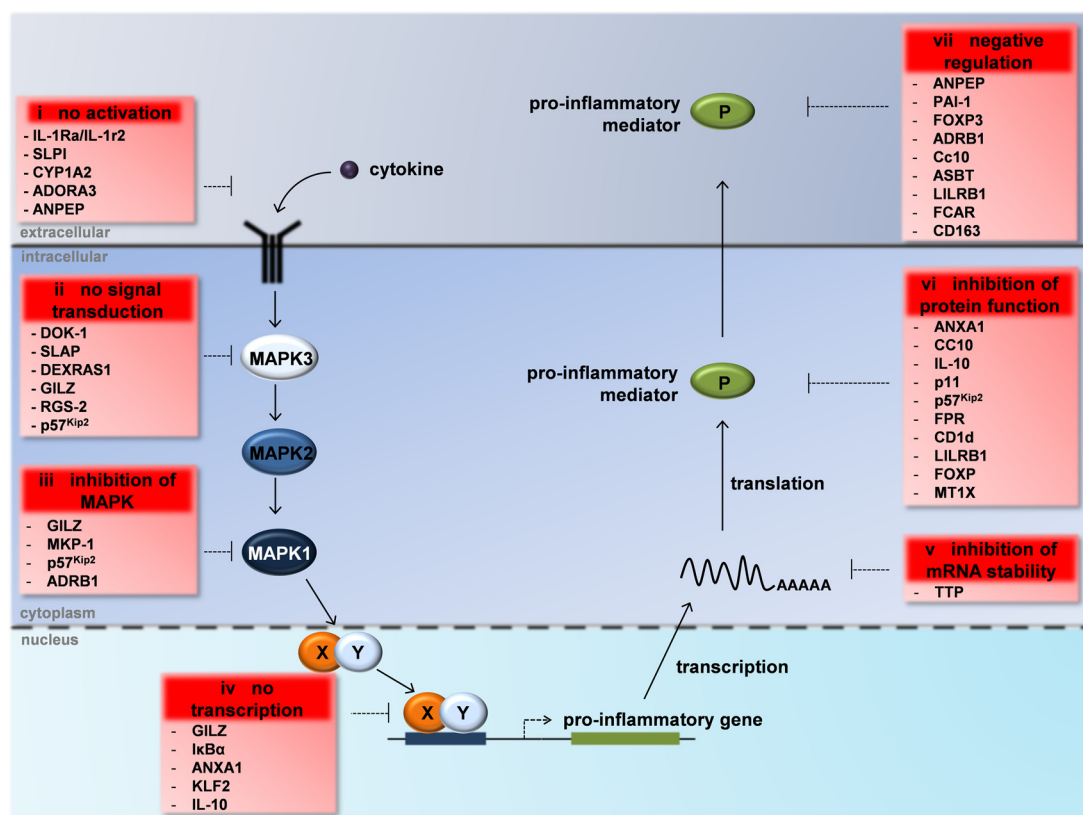
**Table 2.** List of GC-Induced Anti-inflammatory Genes

Symbol	Description	Anti-inflammatory Mechanism	References
<i>ADORA3</i>	Adenosine A3 receptor	Inhibition of eosinophil chemotaxis	(68)
<i>ADRB1</i>	$\beta$ 2-Adrenergic receptor 1	Suppression of JNK signaling, suppression of cytokine secretion	(128)
<i>ANPEP</i>	Aminopeptidase N	Cleaves antigen peptides bound to major histocompatibility complex class II molecules of presenting cells	(129)
<i>ANXA1</i>	Annexin-1	Inhibition of phospholipase 2 (cPLA <sub>2</sub> ), COX-2 and NF- $\kappa$ B	(99–103)
<i>ASBT/SLC10a2</i>	Apical sodium-dependent bile acid transporter	Bile acid transporter	(130–132)
<i>CC10</i>	Clara cell 10 kDa	Inhibition of phospholipase 2 (cPLA <sub>2</sub> ); binds hydrophobic ligands, eg, phospholipids and prostaglandins; inhibits chemotaxis and phagocytosis of neutrophils and monocytes	(133–139)
<i>CD163</i>	Hemoglobin scavenger receptor	Clearance of proinflammatory hemoglobin-haptoglobin complexes	(68, 140–143)
<i>CD1d</i>	Antigen-presenting glycoprotein	MHCI-mediated immunosuppression (stimulates inhibitory NK and invariant T-cells)	(68)
<i>CYP1A2</i>	Thymosin and $\beta$ 4 sulfoxide	Inhibits neutrophil chemotaxis	(144)
<i>DEXRAS1/AGS-1</i>	RAS, dexamethasone-induced 1	Inhibits ligand-dependent signaling by the G <sub>i</sub> -coupled FPR and subsequently ERK-1/2 activation; blocks PKC kinase activity	(145–148)
<i>DOK-1</i>	Docking protein 1	Inhibitory adaptor protein (suppresses activation of MAPK cascade)	(149)
<i>DUSP1/ MKP-1</i>	Dual specificity phosphatase 1	Inhibits MAPKs (JNK/p38/ERK)	(59, 71–75)
<i>FCAR</i>	Receptor for Fc fragment of IgA	Interacts with IgA-opsonized targets	(68)
<i>FOXP3</i>	Forkhead box P3	Suppression of T <sub>reg</sub> cells	(150, 151)
<i>FPR</i>	Formyl peptide receptor	Suppression of cytokine secretion	(68)
<i>IL-10</i>	IL-10	Suppression of T <sub>reg</sub> cells, inhibits expression of proinflammatory cytokines, inhibits NF- $\kappa$ B activation	(68, 152, 153)
<i>IL-1r2</i>	IL-1 receptor type II	Decoy receptor for IL-1 receptor	(154, 155)
<i>IL-1ra</i>	IL-1 receptor antagonist	Competitive inhibition of IL-1b binding to its receptor	(156)
<i>I<math>\kappa</math>B<math>\alpha</math></i>	Inhibitor of NF- $\kappa$ B	Inhibition of NF- $\kappa$ B	(46, 50, 157, 158)
<i>KLF2</i>	Kruppel-like factor 2	Inhibition of NF- $\kappa$ B and AP-1	(159)
<i>LILRB1</i>	Leukocyte immunoglobulin-like receptor, subfamily B member 1	MHCI-mediated immunosuppression	(68)
<i>MT1X</i>	Methallothionein 1X	Free radical scavenger	(160)
<i>p11/ S100A10</i>	S100 calcium binding protein A10	Inhibition of phospholipase 2 (cPLA <sub>2</sub> )	(161, 162)
<i>p57<sup>Kip2</sup></i>	Cyclin-dependent kinase inhibitor 1C	Cyclin-dependent kinase inhibitor	(163)
<i>PAI-1</i>	Plasminogen activator inhibitor 1	Inhibition of the fibrinolytic cascade	(160)
<i>RGS-2</i>	Regulator of G-protein signaling 2	Reduces G <sub>q</sub> -linked signaling	(160)
<i>SLAP</i>	Src-like-adaptor protein	Reduces T-cell signaling by interacting with Syk/Zap70	(164)
<i>SLPI</i>	Secretory leukocyte peptidase inhibitor	Inhibitor of serine proteases	(165)
<i>TSC22D3/GILZ</i>	TSC22 domain family, member 3	Inhibition of NF- $\kappa$ B, AP-1, Raf-1 and Ras	(82–84, 86–90)
<i>TTP</i>	Tristetraprolin	Destabilizes mRNA and increases mRNA turnover	(137, 166–171)

PKC, protein kinase C.

threonine and tyrosine residues in MAPKs, hence the name MAPK phosphatases (MKPs) (69, 70). There are three well-defined MAPK subfamilies: the ERKs, c-Jun N-terminal or stress-activated protein kinases (c-jun N-terminal kinase [JNK] or stress-activated protein kinase), and p38 MAPK. These kinases play an intricate role in the

host-immune response leading to the activation of proinflammatory transcription factors, such as NF- $\kappa$ B and AP-1, and ensuing activation of various cytokines, chemokines, and inflammatory mediators. MKP-1 was originally identified as a phosphatase specific for ERK MAPKs (71, 72). However, consecutive studies have shown that



**Figure 3.** An Overview of All Known GC-Inducible Anti-inflammatory Genes and Their Effects on the Proinflammatory Cascade. GR can resolve inflammation by (i) hampering the activation of proinflammatory signaling pathways through induction of IL-1 receptor antagonist (IL-1ra), IL-1r type II (IL-1r2), secretory leukocyte peptidase inhibitor (Slpi), thymosin  $\beta$  sulfoxide, adenosine A3 receptor (ADORA) and aminopeptidase N (ANPEP); (ii) interfering with signaling cascades through Dok-1, SLAP, Dexras-1, RGS-2, Gilz and p57<sup>Kip2</sup>; (iii) inhibition of subsequent MAPK activation via Gilz, MKP-1, p57<sup>Kip2</sup> and B2 adrenoceptor; (iv) interacting of Gilz, I $\kappa$ B $\alpha$ , Annexin-1, KLF2 and IL-10 with proinflammatory transcription factors; (v) inducing mRNA destabilization through TTP; (vi) inhibiting protein function via induction of Annexin-1, Cc10, IL-10, p11, p57<sup>Kip2</sup>, FPR, CD1d, LILRB1, Foxp3 and MT1X; (vii) negatively regulating various processes through ANPEP, PAI-1, Foxp3, B2 adrenoceptor, Cc10, ASBT, LILRB1, FCAR and CD163.

MKP-1 has a preference for JNK and p38 MAPKs (73–75). The interaction of MKP-1 with its substrates, the MAPKs, increases its activity up to 6- to 8-fold (76). The regulation of *Mkp1* is of much interest but remains controversial. Recently, ChIP sequencing revealed a GRE site in the promoter region of *Mkp1* (77, 78). Moreover, it was shown that the GC-mediated induction of *Mkp1* is dependent on GR dimerization when cells and tissues are exposed to GCs alone or in combination with TNF (10, 59), whereas MKP-1 protein is similarly induced in GC-pretreated cells followed by LPS induction (79). *Mkp1* is expressed in response to GCs in a wide variety of tissues, but it can also be induced by several proinflammatory stimuli, suggesting that MKP-1 functions as a negative feedback regulator of MAPK signaling and is consequently critical for the resolution of inflammation. MKP-1 was also suggested to mediate the protective role of endogenous GCs by interfering with p38 signaling during LPS-induced septic shock (80). Knowledge of the importance of MKP-1 in the combat against inflammation was gained from studies on *Mkp1*<sup>−/−</sup> mice (for an overview of the

use of *Mkp1*<sup>−/−</sup> mice in proinflammatory disease models see Table 3). Additionally, an increasing number of in vivo studies making use of *Mkp1*<sup>−/−</sup> mice demonstrate that MKP-1 contributes to the anti-inflammatory responses of GCs. For example, GCs can protect *Mkp1*<sup>−/−</sup> mice only partly against endotoxic shock (81) and TNF-induced inflammatory shock (59). Mechanistically, MKP-1 protects against TNF-induced lethal shock by dephosphorylating JNK, more specifically JNK-2 (59). Furthermore, it was shown that dimerization of GR is essential for protection against acute TNF-mediated inflammation and critical for *Mkp1* induction and hence controls activation of the proapoptotic JNK-2. In this respect, this study was the first to prove that GR dimerization is also important in the regulation of TNF-induced apoptosis (59).

These findings together show unambiguously that MKP-1 has a pivotal role as a negative feedback regulator of the MAPK-signaling cascade and hence is important in proinflammatory cytokine production and innate immunity.

**Table 3.** Use of *Mkp1*<sup>-/-</sup> Mice in Several Disease Models

Inflammatory Disease Model	Anti-inflammatory Mechanism	Outcome	References
Anaphylaxis	Inhibition of p38	Enhanced mast cell degranulation; increased hypothermia	(172)
Colitis	Inhibition of MAPK	Severe colitis, mucosal hyperplasia	(173))
Diet-induced obesity	Inhibition of JNK	Resistant	(174)
Experimental autoimmune encephalomyelitis (EAE)	Deficiency in CD4 <sup>+</sup> T cells role for JNK?	Resistant	(175)
Experimental induction of arthritis (EIA)	Inhibition of p38	Increased cytokine levels; increased joint-swelling; inflammation in ankle and wrist joints	(176)
Experimental periodontitis	Inhibition of p38	Inflammatory bone loss	(177)
Hypoxia → pulmonary hypertension	Inhibition of p38	Lower levels of eNOS and lower NO production; increased levels of arginase I/II	(178)
Infection with gram-negative bacteria → sepsis	Inhibition of p38 and JNK	Impaired bacterial clearance; increased cytokine levels; infiltration of neutrophils in lungs; increased mortality	(179)
Infection with gram-positive bacteria	Inhibition of p38 and JNK	Increased cytokine and chemokine levels; greater NO production; neutrophil infiltration; severe organ damage; higher mortality	(180)
Influenza viral infection	Defective CD4 <sup>+</sup> /CD8 <sup>+</sup> T cell responses > role for JNK?	Increased weight loss; impaired viral clearance	(175)
Ischemia-reperfusion injury	Inhibition of p38	Greater infarct injury	(181)
LPS-induced endotoxemia and septic shock	Inhibition of p38 and JNK	Increased cytokine levels; hypotension; respiratory failure; increased NO production; MOF; increased mortality	(81, 176, 182–184)
Polymicrobial peritonitis (Casp and CLP)	No mechanism described	Increased cytokine and chemokine levels; increased lethality	(185)
Stress	Inhibition of p38 and JNK		(184, 186)
TNF-induced acute inflammation	Inhibition of JNK-2	Increased cytokine and chemokine levels; enhanced intestinal damage; increased mortality; cell death	(59)
Zymosan-induced inflammation	Inhibition of p38 and JNK	No response to Dex in terms of leukocyte infiltration and cytokine suppression	(79)

CLP, cecal ligation and puncture; Dex, dexamethasone; eNOS, endothelial NOS; MOF, multiple organ failure.

### GC-induced leucine zipper (GILZ)

*Tsc22d3* (encoding GC-induced leucine zipper or GILZ) is considered a prototype of a GC-induced gene and is therefore often represented as a mere readout product of the GC-induced signaling cascade. However, it also mediates the effects of GCs in immune function. GILZ belongs to the family of TGF- $\beta$ -stimulated clone 22 domain (TSC22D) proteins. This family includes genes transcriptionally activated by TGF- $\beta$  and GCs in a wide variety of cell lines and tissues (82–84). *Tsc22d3* induction by GCs is inhibited in GR<sup>dim/dim</sup> mice (Ref. 85; our unpublished results). Moreover, the *Tsc22d3* promoter region displays six putative GRE motifs, as well as motifs for other transcription factors. The GILZ protein has been reported to bind to Ras and Raf-1 and the downstream proinflammatory transcription factors NF- $\kappa$ B and AP-1 (86, 87). Ras is a membrane-associated protein activating a number of signaling cascades, including the RAF-

MEK-ERK and phosphatidylinositol-3 kinase-AKT pathways (88–90). Furthermore, by binding to Raf-1, GILZ inhibits MEK and ERK phosphorylation and subsequent activation. In this way, GILZ induction seems to be one of the mechanisms by which GCs regulate the MAPK-signaling cascade, albeit indirectly. Next, GILZ has also been shown to interact with p65 (subunit of NF- $\kappa$ B) and both c-Fos and c-Jun (subunits of AP-1) (86, 91). These anti-inflammatory properties of GILZ indicate an immune modulatory role. The anti-inflammatory actions of GILZ have been confirmed by using mouse models of chronic inflammatory diseases, such as dinitrobenzene sulfonic acid (DNBS)-induced colitis (a model of inflammatory bowel disease) (92), collagen-induced arthritis (CIA) (a murine model of RA) (93), and experimental autoimmune encephalomyelitis (a model of multiple sclerosis [(MS)]) (94). The use of GILZ-overexpressing transgenic mice demonstrated that GILZ can antagonize the development



of colonic inflammation induced by DNBS (92). In addition, in vivo delivery of *Tsc22d3* small interfering RNA in CIA mice increased disease severity, indicating that GILZ has an important protective function (93). Moreover, in vitro, GILZ small interfering RNA inhibited the suppression of LPS-induced cytokines by GCs (95). Furthermore, GILZ administration had a more protective effect than the administration of high doses of GCs in both DNBS-induced colitis and CIA. In addition, the anti-inflammatory actions of GCs (up-regulation of GILZ upon GC treatment has also proven effective) in patients suffering from alcoholic hepatitis (AH) are dependent on Gilz (96). In summary, these data show that GILZ is a key mediator of the anti-inflammatory properties of GCs.

### Annexin-1

Annexin-1 or lipocortin-1 (encoded by *AnxA1*) is a member of the annexin superfamily of calcium- and phospholipid-binding proteins (97). The human *AnxA1* promoter region contains a GRE element, but whether it can be induced in GR<sup>dim/dim</sup> mice has not been reported (98). Annexin-1 was originally described as a GC-induced protein inhibiting the activity of phospholipase A<sub>2</sub>, which is known to cleave arachidonyl-containing phosphatides in the cell (99, 100). Arachidonic acid can be further modified by cyclooxygenases (COX) to yield the proinflammatory mediators prostaglandins and leukotrienes. Annexin-1 also inhibits NF- $\kappa$ B, by binding to the p65 subunit and thereby prevents its binding to DNA and to COX-2 (101–103). Neutralizing antibodies against annexin-1 abrogated the inhibitory action of GCs in the rat hind paw carrageenan edema model and in a rat ischemia-reperfusion injury model (104). Studies on *AnxA1*<sup>−/−</sup> mice showed that annexin-1 is protective in AIA, bleomycin-induced lung fibrosis, and dextran sodium sulfate-induced colitis: the diseases were more severe in *AnxA1*<sup>−/−</sup> mice (105–107). It has also been suggested that annexin-1 is protective in CIA, ulcerative colitis, and chronic granulomatous inflammation (102, 108, 109). Moreover, GCs exerted no inhibitory effects in *Anxa1*<sup>−/−</sup> mice in a carrageenan- or zymosan-induced inflammatory model or in AIA, suggesting that annexin-1 mediates anti-inflammatory actions of GCs (107, 110). Annexin-1 was also shown to modulate the repair of gastric mucosal injury, because treatment with an annexin-1 mimetic significantly enhanced gastric ulcer healing (111) and the use of an annexin-1-based peptide, MC-12, resulted in amelioration of symptoms in both dextran sodium sulfate and 2,4,6-trinitrobenzene sulfonic acid-induced colitis models in mice (112). In summary, these findings raise interest in annexin-1 as a GC-inducible effector of inflammation resolution.

### GR-mediated TR of nGRE genes

Figure 2 explains how GR dimers are also required for TR of nGRE genes (33, 34). These so-called nGRE elements, comparable to normal GRE, are composed of two inverted repeats (hexanucleotides) that are either adjacent or separated by one or 2 bp (CTCC(n)<sub>0–2</sub>GGAGA; referred to as IR0, IR1 and IR2, respectively) (35). However, the anti-inflammatory capacity of GR-mediated TR of nGRE genes is unknown. Nevertheless, a recent study by Surjit et al. (35) indicated that TR of nGRE genes by GR dimers can transcriptionally repress the expression of the cytokine thymic stromal lymphopoietin through direct binding of dimeric GR to a nGRE. This mechanism could account for the GR-mediated restriction of atopic dermatitis. These findings suggest that the sensitivity of GR<sup>dim/dim</sup> mice in several disease models can also be accounted for by reduced TR of nGRE genes. It was reported that nGREs are present in more than 1000 mouse/human ortholog genes, some of which are known to encode proinflammatory mediators, which indicates the importance of this mechanism as an additional level of anti-inflammatory GR signaling (35). The contribution of nGRE genes to the anti-inflammatory cascade of GR remains to be elucidated, but nevertheless poses an interesting field of investigation. Thorough investigation, for example by studying the expression profiles of these genes in GR<sup>dim/dim</sup> mice, could lead to the identification of new anti-inflammatory GR targets.

### GC-mediated proinflammatory effects

The above-mentioned studies demonstrate the strong anti-inflammatory actions of GCs. However, GCs are not exclusively immunosuppressive (113); GCs also assist in maintaining and even facilitating immunity. For example, adrenalectomized mice and patients with Addison's disease produce no GCs, and both of these are more susceptible to infection (114). Indeed, it has been reported that GCs can have enhancing effects on immune cells (115). For example, it has been reported that depending on the composition of the GR-AP-1 dimer, GR can influence the activity AP-1 either positively or negatively (116). Also, disruption of GC action in osteoblasts resulted in a more rapid resolution of inflammation in the K/BxN model of experimental arthritis, suggesting that GCs have a proinflammatory role in this model (117). Moreover, in addition to their immunosuppressive effect on TLR signaling, GCs also affect TLR expression. For instance, the promoter of TLR2 is cooperatively stimulated by GCs and TNF, through the presence of a functional NF- $\kappa$ B site, a GRE element, and a signal transducers and activators of transcription-binding element (118). Generally, GCs are immune stimulatory within the normal physiologic range

of hypothalamic-pituitary-adrenal axis activity and inhibitory when GC levels are higher, as in chronically stressed animals. These findings clearly indicate the effects of GCs are critically dose dependent: supraphysiologic doses of GCs most probably result in the widely GC-mediated anti-inflammatory effects, whereas lower doses can be immunomodulatory.

## Conclusion and Future Perspectives

In this review we emphasize the importance of GR dimerization in the combat against, or resolution of, inflammation. It is generally believed that the anti-inflammatory aspect of GR results from TR of proinflammatory genes by the tethering of monomeric GR to other transcription factors. However, some recent studies using GR<sup>dim/dim</sup> mutant mice indicate that GR dimers also account for the resolution of inflammation by GR. The physiology of GR, ie, its isoforms, posttranslational modifications, the recruitment of cofactors, and its subsequent actions are strongly tissue specific. Moreover, there is substantial temporal variation in GC-mediated actions, and this is reflected in time-dependent gene-specific induction. This might explain the discordant reports on the response of GR<sup>dim/dim</sup> mice in distinct inflammatory environments (Table 1). It is worthwhile to decipher the tissue- and time-specific effects of GCs because it could resolve the contradictions in the reported results and clarify the role of GR actions in several diseases.

Interestingly, the continuing identification of new GRE-dependent genes with anti-inflammatory properties demonstrates that the TA potential of GR is indispensable and indicates that the mechanism of the anti-inflammatory action of GR is far from completely understood, including the unidentified role of nGRE-dependent genes. In-depth knowledge of these mechanisms will elucidate whether GR dimerization preventing GR ligands are, in fact, potential therapeutics in the combat against inflammation or might be dangerous rather than helpful in this aspect. Indeed, many scientists have tried to develop SEGRAs that preferentially induce the formation of monomers (33, 35, 42, 119–121). However, only two compounds have made it to clinical trials for topical application. This is probably due to the fact that an increasing amount of data is being published on the importance of GR dimers in the resolution of inflammation. Furthermore, the above-mentioned dogma is challenged by data showing that GR<sup>dim/dim</sup> mice still suffer from some side effects upon GC treatment (122), which means that not all side effects can be explained by reduced GR TA activity. Although it was demonstrated that GR dimers play an

intricate role in the development of hyperglycemia and wound repair (123, 124), GC therapy in GR<sup>dim/dim</sup> mice still reduces bone formation and attenuates osteoblast differentiation, both of which are characteristics of GC-induced osteoporosis (85, 123–125). Next, GR<sup>dim/dim</sup> mice and GR<sup>wt/wt</sup> mice show the same degree of muscle atrophy upon GC therapy, suggesting that monomeric GR is sufficient to cause skeletal muscle atrophy (126). This could be because not all genes that are positively regulated by GR are affected by the GR<sup>dim/dim</sup> mutation, such as genes dependent on composite elements or tethering mechanisms (Figure 2). It must be noted that it is difficult to differentiate between TA and TR because the GR coactivator GR-interacting protein 1 is also recruited to sites of GR repression, indicating that it also has a corepressor function (20). These findings indicate that GR-interacting protein 1 has a dual function: facilitating both TA and TR aspects of GR action depending on the genomic context. This indicates that dissociating compounds will likely still induce certain unwanted side effects. In addition, SEGRAs might not activate all the mechanisms of TR actions. For example, compound A effectively blocks NF- $\kappa$ B, but not AP-1 (Ref. 127 and our unpublished data). Moreover, an emerging role for GR-dimer-mediated TR of nGRE genes also questions the use of SEGRAs. So far, the molecular mechanisms of GR-induced restriction of inflammation are not completely understood and pose an interesting field of investigation. In-depth knowledge of these mechanisms will elucidate whether GR ligands or SEGRAs are potential therapeutics for inflammation, or whether they could be dangerous because they might cause immunostimulation in certain inflammatory diseases. Here, we want to stress that thorough studies are needed to unravel the mechanistic details of the anti-inflammatory cascade of GR, in an inflammation-specific way. Hence, the identification and further use of SEGRAs obviously hold a brake to the full cascade. The identification of disease-specific GR agonists will be necessary to reduce patient suffering and decrease economic costs.

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Address all correspondence and requests for reprints to: Claude Libert, VIB-Department for Molecular Biomedical Research /Ugent, Technologiepark 927, Zwijnaarde 9052, Belgium. E-mail: Claude.libert@dmbr.vib-ugent.be.

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